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Regular paper

The effect of bisphenol A on growth, pigment composition and photosystem II activity of *Arabidopsis thaliana**

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Bisphenol A (BPA) is a widely used chemical, that can potentially be toxic to plants. In this study we examined the toxicity of 5–50 mg/l of BPA on *Arabidopsis thaliana*. Additionally, the effects of 0.5–5 mg/l of BPA were examined after four weeks of development. BPA had no effect on the germination rate and the chlorophyll *a/b* ratio. The chlorophyll *a* and carotenoid content was significantly elevated in seedlings treated with 5 mg/l of BPA. In 4-week-old plants there was no change in the chlorophyll and carotenoid content and photosynthetic parameters (F_v/F_m, F_v/F_0 and PI) were unaffected, which suggests no photoinhibition. No oxidative stress symptoms were observed. BPA significantly decreased leaf protein content. A low concentration of BPA seems to have no significant effect on *A. thaliana* flowering, but further investigation is needed. The results obtained indicate that a low concentration of BPA has no negative effect on the growth and development of *A. thaliana*.

Key words: BPA, bisphenol A, *Arabidopsis thaliana*, seedlings, plants, chlorophyll

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Abbreviations: BPA, bisphenol A; 2,2-bis(4-hydroxyphenyl)propane; Chl, chlorophyll; F_0, initial chlorophyll fluorescence intensity measured in PSII; F_m, maximal chlorophyll fluorescence intensity; F_v, variable chlorophyll fluorescence ($F_m - F_0$); F_v/F_m, maximal quantum efficiency of photosystem II; F_v/F_0, oxygen-evolving complex efficiency; MDA, malondialdehyde; PI, performance index; PS II, photosystem II

INTRODUCTION

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane or BPA) is a chemical additive (Fig. 1) widely used in the production of carboxyplastics. It can be found in plastic bottles, notebooks and some paints (Goodson *et al.*, 2002; Burridge 2003). As plastic ages some monomers of BPA can leak to the environment. This contamination is believed to be potentially harmful for living organisms. BPA mimic estrogenic activity, acting as an endocrine disruptor and can cause fertility problems (Mandich *et al.*, 2007; Molina *et al.*, 2013). Early-life BPA exposure may increase the risk of breast cancer in animal model (Seachrist *et al.*, 2016). BPA may affect human reproductive system and might promote prostate cancer (Tarapore *et al.*, 2014). BPA is also linked with obesity and diabetes (Somm *et al.*, 2009; Ranciere *et al.*, 2015).

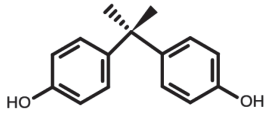

Figure 1. Chemical structure for BPA (drawn using ChemSpider).

Fortunately, BPA is easily biodegradable by microalgae (Gattullo *et al.*, 2012) and microorganisms present in water and soil (Matsumura *et al.*, 2015; Ren *et al.*, 2016). On the other hand, the prevalence of plastic pollution is a serious risk of continuous low-dose BPA exposure.

Scientific data regarding the toxic effect of BPA on plants is still scarce. It is known that some plants rapidly metabolize and absorb BPA in the form of glucoside conjugates (Nakajima *et al.*, 2002). It was shown that a high concentration of BPA (above 10 mg/l) has a negative effect on the growth and development of many plant species, including some important crops e.g. soybean (Qiu *et al.*, 2013). In contrast, according to our knowledge low-dose effects of BPA on pigment composition in *Arabidopsis thaliana* have not been the subject of detailed studies. The impact of BPA on plants may be as complex as in animals, since mammal sex hormones are also produced by plants (Janeczko *et al.*, 2005) and estrogen receptors have been found in plants (Janeczko *et al.*, 2008). Thus, BPA can affect plants in both ways – as a toxic agent or by affecting particular estrogene-related regulatory pathways.

We were particularly interested in the low-dose and long-term effects of BPA on development of *A. thaliana*. BPA is a ubiquitous pollutant. However, in most cases its concentration in the environment is relatively low. Nevertheless, this does not mean such a concentration is safe for plants. Relatively few studies were done using plants older than one week. We checked the effect of a wide range of BPA concentrations on seedling pigment composition. The upper concentration of BPA was chosen arbitrarily, according to the concentrations described in other articles. We found a low concentration of BPA, which was particularly interesting, because we observed some simulative effects on pigment composition and no obvious stress symptoms. Then we checked the long-term (after four weeks) effects of BPA on the growth of *A. thaliana*, including changes in photosystem II (PSII) activity, stress symptoms as well as pigment composition.

MATERIALS AND METHODS

Plant material. *Columbia* ecotype (Col-0) of *A. thaliana* was used in all experiments. The seeds were obtained from Arabidopsis Biological Center (Ohio State University, Columbus, OH, USA). All of the seeds used in the experiments were collected from plants grown

under standardized conditions, 12L:12D photoperiod at light intensity of 70–100 $\mu mol \times m^{-2} \times s^{-1}$ (Sylvania, Luxline plus), 80% humidity, and at 22±2°C.

Medium preparation and growth conditions. The seeds were sterilized for 7 mins with 3% hyperchlorite with an addition of 0.1% of Triton X-100 (Serva, Germany), washed thoroughly with sterile, deionized water and sown on glass Petri dishes or glass jars containing a Murashige and Skoog medium (Duchefa) with 1% agar and 3% sucrose (Sigma-Aldrich), as described in Malec *et al.* (2002). The growth medium was supplemented with sterile (filtered through a Whatman 0.2 μm nylon filter) bisphenol A (Sigma-Aldrich) dissolved in 96% ethanol (to a final concentration of 0.5; 1; 5; 25 and 50 mg/l). For the control experiments, ethanol was added to the medium (0 mg/l of BPA) in order to eliminate inequality caused by the solvent. The ethanol concentration in the medium did not exceed 0.05% (v/v) of the final growth medium volume. To avoid additional, unintentional BPA contamination all experiments were conducted using nitrile gloves and labolatory glasswere. The plants were placed at 4°C/48 h for stratification, then grown in white light (Sylvania, Luxline plus; fluence rate 80–90 $\mu mol \times m^{-2} \times s^{-1}$) at 22±2°C and 16L/8D photoperiod for 1 week or 4 weeks.

Calculating the germination rate. The seedlings with visible cotyledons were manually counted and divided by the number of sown seeds.

Chlorophyll *a* fluorescence and photosystem II activity determination. Handy PEA fluorimeter (Hansatech Instruments) was used for estimating the photosynthetic parameters (including F_v/F_m, F_v/F_0, PI) in four-week old leaves. Before measuring, the plants were dark-adapted for 15 minutes.

Pigment composition. The chlorophyll and carotenoid content were estimated in 80% (v/v) acetone extracts according to Lichtentaler (1987). The antocyanin content was measured spectrophotometrically accroding to Muravyeva and coworkers (1984). 70–100 mg of plant material (seedlings or leaves) was used for each extraction.

Antioxidative potential. Extracts of powdered leaves from the four-week-old plants were obtained using methanol solution. EPR-based radical scavenging of

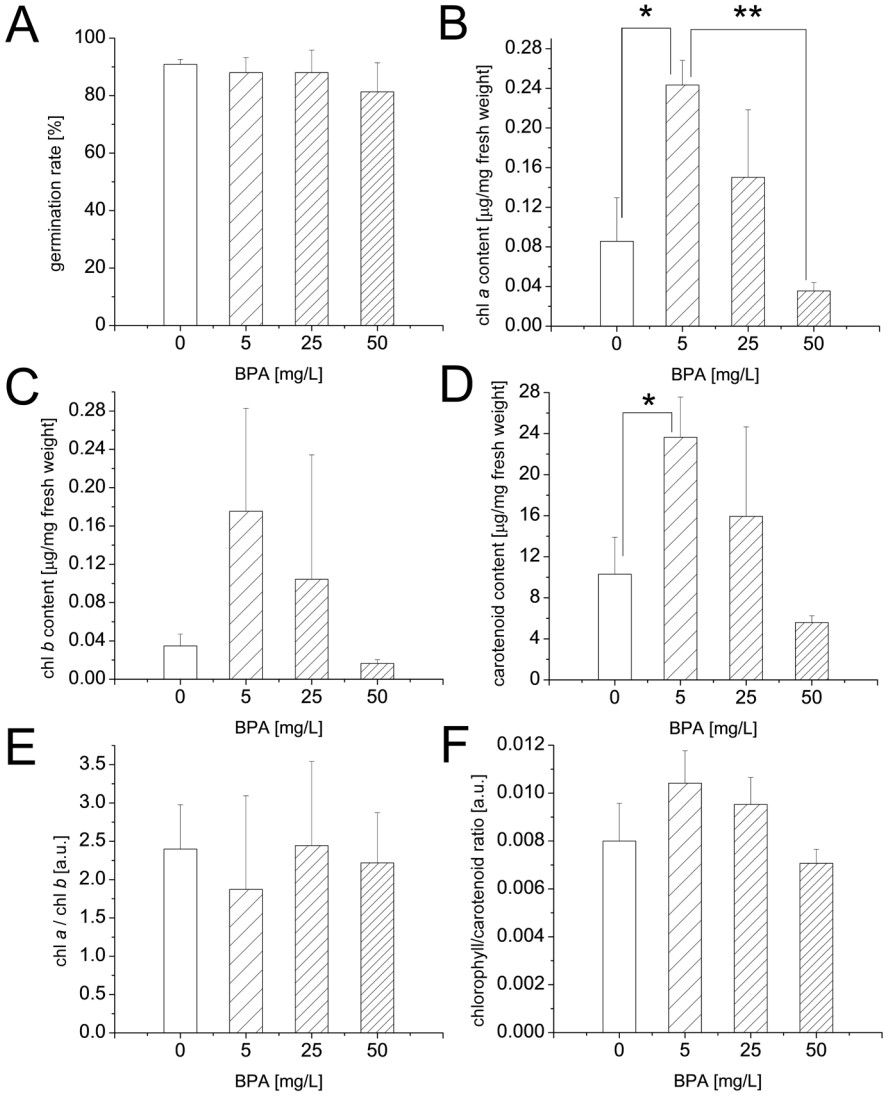

Figure 2. Effect of BPA-treatment on germination rate and pigment composition in one-week-old *A. thaliana* seedlings.
(**A**) *in vitro* germination rate of *A. thaliana* ecotype *Columbia* after one week in presence of 0, 5, 25 or 50 mg/l of BPA. (**B**) Chl *a*, (**C**) chl *b*, (**D**) carotenoid content, (**E**) chl *a*/*b* ratio and (**F**) chl/car ratio in seedling extracts. Germination rate was estimated using at least 100 seeds. All experiments were repeated four times. Significance was indicated with stars *$p<0.05$; **$p<0.01$. Error bars – standard deviation.

the extracts was carried out using 0.3 mM α-diphenyl-β-picrylhydrazyl (DPPH; Sigma-Aldrich). The spectra were measured at room temperature, using a MiniScope MS300 (Magnettech GmbH) spectrometer with a frequency of 9.4 GHz, amplitude modulation of 2000 mG, microwave power of 10 mW and scan rate 42 g/min (noise was reduced by averaging 6 scans for each sample).

Thiobarbituric acid reactive substances (TBARS) assay. The MDA content was measured spectrophotometrically using ethanol-based extracts of four-week-old leaves, according to the method described by Hodges and coworkers (1999).

Protein content. 50 mM phosphate buffer (pH 7.5) was used to extract soluble protein. The estimates were made spectrophotometrically using Bradford reagent (Sigma-Aldrich) and bovine serum albumine (Sigma-Aldrich) for a standard curve (Bradford, 1976). The samples were incubated per 15 minutes at 20±2°C. All measurements were taken within the next 10 minutes.

Statistical analysis and normalization. The pigment content, antioxidative potential, protein and MDA content were normalized to fresh weight. The statistical significance was estimated by analyzing the variance (one-way ANOVA), followed by two-tailed Tukey's post-hoc test. *P*-values less than 0.05 were considered to be significant. All analyses were carried out in R version 3.2.4 (codename "Very Secure Dishes") on Ubuntu. Graphs and correlation coefficient were produced using Origin 6.0 (OriginLab, Northampton, MA).

RESULTS AND DISCUSSION

The effect of BPA on the early stages of *A. thaliana* development

After one week of exposure BPA had no significant effect on the germination rate (Fig. 2A). This is consistent with previous observations regarding *A. thaliana* (Pan *et al*., 2013) and other species (Ferrara *et al*., 2006; Dogan *et al*., 2010; Dogan *et al*., 2012).

BPA had an adverse effect on chlorophyll *a* and the total carotenoid content. 5 mg/l of BPA significantly

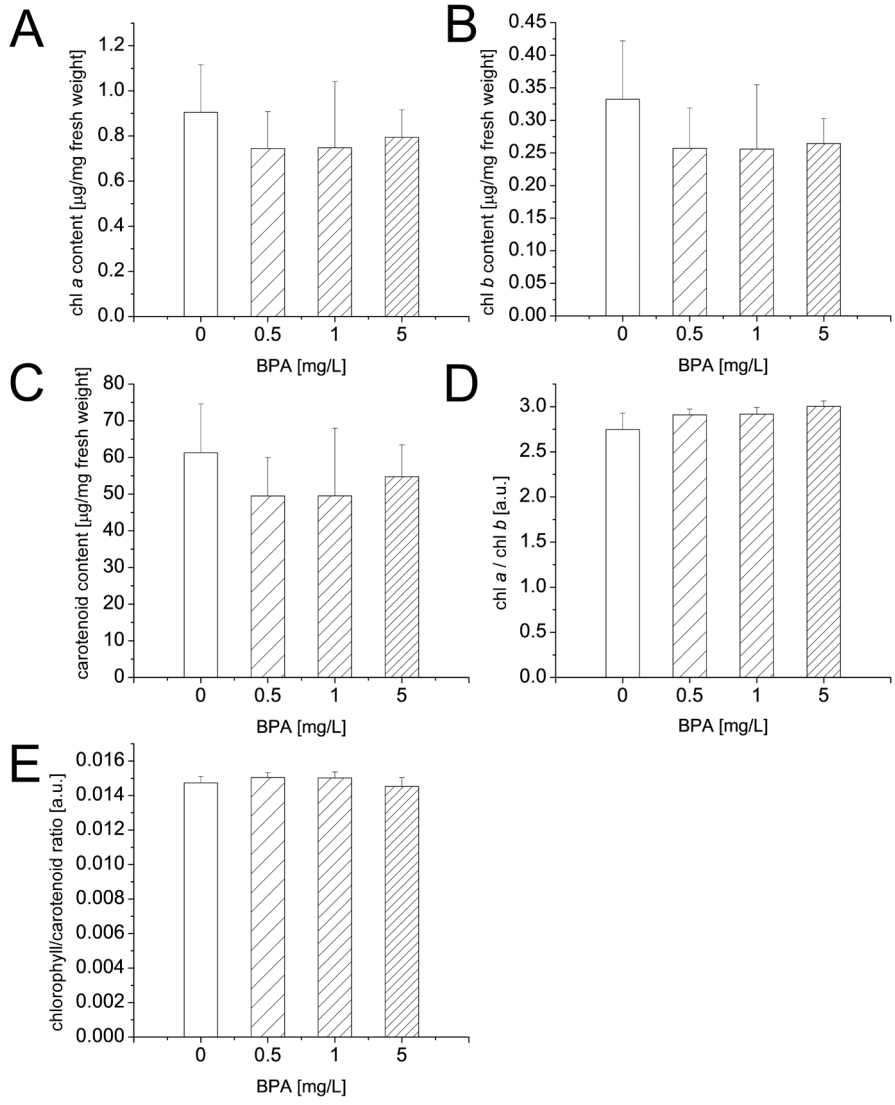

Figure 3. Effect of BPA-treatment on pigment composition in 4-week-old *A. thaliana*.
(**A**) Chl *a*, (**B**) Chl *b*, (**C**) carotenoid content (**D**) chlorophyll *a*/*b* ratio, (**E**) chl/car ratio in extracts from leaves of 4-week-old plants. All experiments were repeated four times. Error bars – standard deviation.

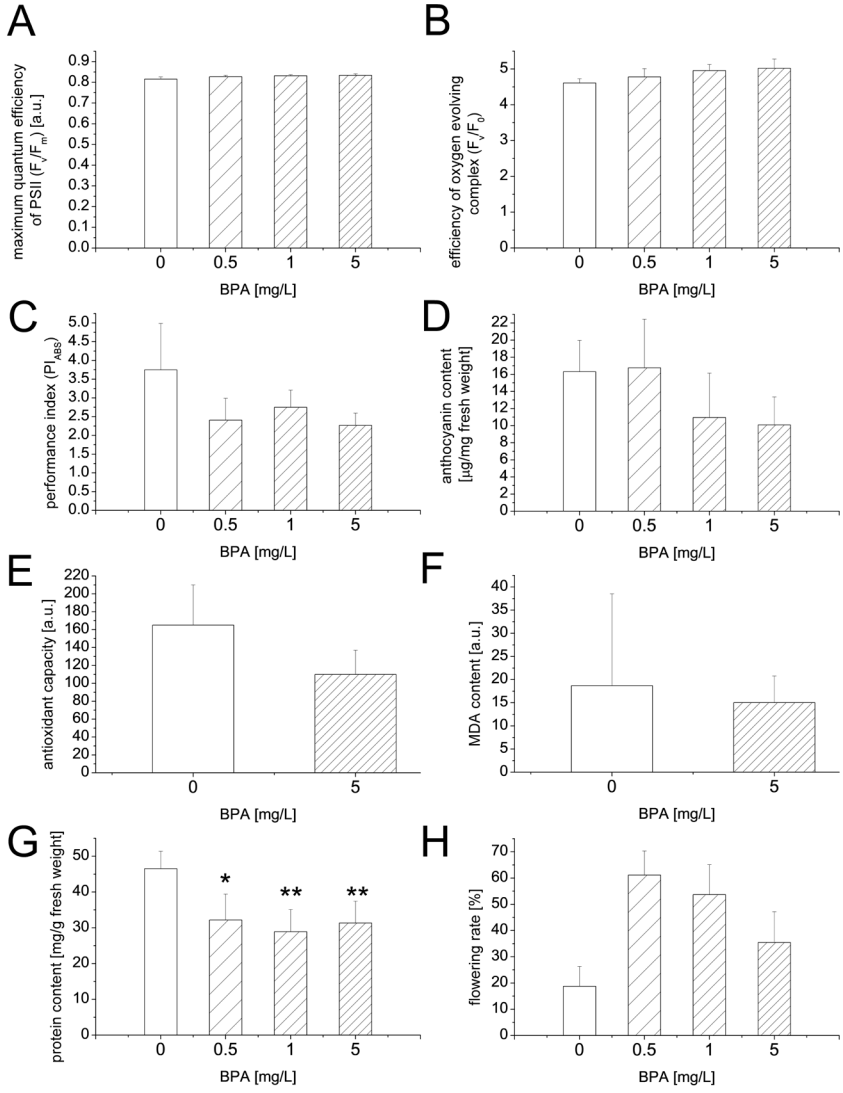

Figure 4. Physiological and biochemical characteristics of BPA-treated 4-week-old *A. thaliana*
Photosynthetic parameters (**A**) F_v/F_m, (**B**) F_v/F_0 and (**C**) PI estimated for 4-week-old BPA-treated plants. (**D**) Anthocyanin content, (**E**) DPPH-based EPR study of antioxidant potential, (**F**) MDA content in extracts from *A. thaliana* treated with 0 and 5 mg/l of BPA. (**G**) Protein content in plants treated with 0, 0.5, 1 and 5 mg/l of BPA. Significance in comparison to control plants is indicated with stars *p<0,05; **p<0,01. All experiments were repeated four times. Error bars – standard deviation. (**H**) Percentage of flowering 4-week-old *A. thaliana* plants. Error bars – standard error.

doubled chlorophyll *a* and carotenoid content in seedlings treated (Fig. 2B and 2D). Higher concentrations of BPA (25 and 50 mg/l) decreased the mean chlorophyll and carotenoid content in comparison to plants treated with 5 mg/l of BPA, but the differences were statistically insignificant in comparison to control plants. However, the difference in chlorophyll *a* content between plants treated with 5 and 50 mg/l of BPA was statistically significant. Moreover, these changes in the chlorophyll *a*, *b* and carotenoid content were dose dependent and inversely proportional to the BPA concentration. However, it must be emphasized, that chlorophyll *b* content in plants treated with 25 mg/l of BPA varied greatly with no obvious outliers. Thus, these observations may be subject to considerable uncertainty. Nevertheless, BPA seems to have an adverse effect on chlorophyll accumulation. Treatment with 5 mg/l of BPA apparently stimulates chlorophyll accumulation but a higher BPA concentration decreases chlorophyll accumulation. The stimulatory effect of BPA on chlorophyll accumulation in *A. thaliana* has not been previously observed. However, a low BPA concentration (corresponding to approximately 0.2 and 1 mg/l) has been shown to increase the fresh weight and promoted root development of *A. thaliana* seedlings, while higher BPA concentrations had the opposite effect (Pan *et al.*, 2013). The stimulatory effect of low BPA-concentration on chlorophyll accumulation has been observed in other plants, especially in soybean seedlings (Hu *et al.*, 2014). BPA (1.5 mg/l) increases the activity of five enzymes, involved in chlorophyll biosynthesis (Jiao *et al.*, 2015; Jiao *et al.*, 2017). The positive effect of BPA on chlorophyll accumulation is probably not a result of its estrogenic activities, as low-dose heavy metal concentrations also increase chlorophyll content in plants (Maleva *et al.*, 2009; Kumar *et al.*, 2015). In our study the stimulatory effect of BPA on chlorophyll accumulation was observed at 5 mg/l of BPA. No change in the chlorophyll to carotenoid ratio (considered as a stress indicator in *A. thaliana* which correlates with photoinhibition) suggesting, that the physiology of *A. thali-*

ana seedlings was not negatively altered by BPA in these conditions (Fig. 2F). This may indicate a high level of tolerance to BPA pollution (thus, its relatively low toxicity) in the early stages of *A. thaliana* growth. BPA had no significant effect on the chlorophyll *a*/*b* ratio (Fig. 2E) and apparently does not affect the PS I and PS II antenna stoichiometry in seedlings.

The effects of BPA-treatment on *A. thaliana* after 4 weeks of development

Measuring chlorophyll *a* fluorescence *in vivo* is an important tool for estimating photosynthesis-related parameters. In dark adapted leaves a millisecond flash of light induces rapid fluctuations in chlorophyll fluorescence, which is correlated with photochemical reactions during photosynthesis. As a result, the characteristic OJIP curve of light emission can be recorded, as reviewed in Misra and coworkers (2012). The initial chlorophyll fluorescence yield, F_0, can be recorded after 20 μs upon excitation with photosynthetically inactive light and represents conditions with fully oxidized plastoquinone pool. The second pulse of photosynthetically active light induces intense fluorescence, described as F_m and which represents the maximal fluorescence yield in the absence of photochemical quenching. F_m is reached when the primary electron acceptor pool in PS II is fully reduced and energy transfer from the excited chlorophyll is impossible. A comparison of these values, expressed as $(F_m - F_0)/F_m$ or F_v/F_m ratio allows us to estimate the maximal quantum efficiency of PS II. Other parameters can be described using a OJIP curve, including oxygen-evolving complex efficiency (F_v/F_0) and performance index (PI) (Maxwell *et al.*, 2000; Misra *et al.*, 2012; Kalaji *et al.*, 2014).

In our study BPA did not affect PSII activity in *A. thaliana*, as F_v/F_m, F_v/F_0 and PI were not significantly altered (Fig. 4A, 4B and 4C). Therefore, BPA caused no photoinhibition. It was observed, that low doses of BPA may even stimulate photosynthesis and increase F_v/F_m in seedlings of particular crops, but the effect was dependent on BPA concentration and the species susceptibility (Zhang *et al.*, 2015).

BPA treatment had no effect on plant size and BPA had no significant effect on the chlorophyll (Fig. 3A and 3B) and carotenoid (Fig. 3C) content in *A. thaliana*. Our observations suggest that BPA had no effect on PS I and PS II antenna stoichiometry in 4-week-old plants, as no change in the chlorophyll *a*/*b* ratio was observed.

BPA had no significant effect on the anthocyanin content (Fig. 4D) and chlorophyll/carotenoid ratio (Fig. 3E). No significant change in antioxidative potential was observed either (Fig. 4E). Additionally, MDA content was also similar in BPA-treated and control plants (Fig. 4F), but these results may be subject to considerable uncertainty. Nevertheless, no significant oxidative stress symptoms were observed, indicating that low-dose (up to 5 mg/l) BPA pollution may be relatively safe for plants. Our results contradict some other findings, where BPA exposure had a severe effect on *A. thaliana* development (Tian *et al.*, 2014). Accroding to this research, the minimal, considerably toxic, BPA concentration for *A. thaliana* exceeds 10 mg/l (50 μM). Such a BPA concentration is observed in heavily polluted and environmentally devastated, industrial areas (Yamamoto *et al.*, 2001). However, much lower concentration of BPA in soil and water is more probable in agricultural fields. In German rivers the BPA concentration was estimated to be about 0.41 mg/l (Fromme *et al.*, 2002). Our data suggest that low-dose BPA exposure may not be directly dangerous to plants that grow in non-industrial areas and in agricultural fields, polluted only by typical plastic waste.

Although we found no statistically significant differences in antioxidant potential and anthocyanin content, it is worth emphasizing that BPA-treated plants had a lower mean content of antioxidants. Thus, low-dose BPA-treatment seems to decrease antioxidant capacity in some plants. It seems possible that even a low concentration of BPA had some negative effect on particular individuals in the population. It may be possible that some of defence mechanisms were weakened by BPA, making these plants more susceptible to other environmental stresses. In optimal laboratory conditions, differences might be meaningless, but in wild, polluted population can make a difference. Furthermore, in an agricultural field even low-dose BPA pollution may decrease plant yields. This is an important issue, especially considering the gradual decrease of protein content in BPA-treated plants. Protein content was significantly lowered in BPA-treated plants (Fig. 4G) and these results were dose-dependent. Leaf protein content seems to correlate with yield quality and protein content in the seeds of legumes (Nielsen *et al.*, 1994). Apparently, no information about the effect of BPA on the nutritional value of legume seeds is available. Importantly, nitrogen deficiency may decrease the soluble protein content in particular plants e.g. sunflowers (Pankovic *et al.*, 2000). However, such an effect was not observed in *A. thaliana* but the yield (number of seeds) was reduced in those conditions (Lemaître *et al.*, 2008). 1.5 mg/l of BPA stimulates nitrogen accumulation in soybean (*Glycine max*) roots and the seedlings displayed higher fresh and dry weight (Sun *et al.*, 2013). Higher concentrations of BPA had the opposite effect. Interestingly, a low concentration of exogenous estrogens had a similar effect on the roots of *A. thaliana*, increasing the protein content and dry mass (Kopcewicz *et al.*, 1970a). BPA may have a similar, regulatory effect on the early stages of the growth of *A. thaliana*. Therefore, it is possible, that intense nitrogen assimilation at an early stage of growth may reduce its content in the medium, leading to its being insufficiently supplemented during later stages. As protein content was measured in leaves only, it is possible, that BPA treatment may change nitrogen allocation between the shoot and root. The nitrogen shoot:root ratio allocation depends on nitrogen abundance, as shown in many crops (Lemaître *et al.*, 2008). On the other hand BPA may affect N partitioning between protein and pigment biosynthesis (as chlorophyll content was increased in seedlings). Therefore, the allocation of nitrogen in the chlorophyll pool reduces its availability, as chlorophyll and products of its degradation generally not release this macronutrient. Thus, the nitrogen pool for protein recirculation might be reduced in BPA-treated plants, especially at a later stage of development. Most of the cellular proteins are involved in photosynthesis, of which RuBisCO makes up up to 50% of soluble proteins (Spreitzer and Salvucci, 2002). Therefore, the reduced soluble protein content may be considered to be a reduced RuRisCO content (Mate *et al.*, 1993) and perhaps activity too. However, even a 50% reduction in the normal RuBisCO content had no visible effect on the photosynthesis rate and CO_2 assimilation (Quick *et al.*, 1991). As carbon and nitrogen metabolism are co-regulated (Ferrario-Méry *et al.*, 1998), the change in protein content may reflect altered carbohydrate accumulation as well as the content of soluble nitrogen-containing compounds (and water content) (Neto *et al.*, 2009).

The decreased protein content (associated with nitrogen deficiency) has little impact on the F_v/F_m ratio in maize and the first negative effects occurred in 5-week-old plants (Ding *et al.*, 2005). Thus, the decreased protein content in *A. thaliana* may have no visible trace in plant development and its negative effects may be delayed.

The leaf protein content correlates negatively with the chlorophyll *a*/*b* ratio in some plants, especially in high light conditions (Kitajima *et al.*, 2003). Although in our experimental model the mean values of the chlorophyll *a*/*b* ratio were slightly increased in BPA-treated plants, the difference was insignificant (Fig. 3D) and had no correlation to BPA concentration.

Apparently, the decreased soluble protein content did not indicate stress in BPA-treated plants, because the protein content in plants exposed to different types of stress conditions usually increases or no significant change was observed (Gonçalves *et al.*, 2007; Neto *et al.*, 2009; Azymi *et al.*, 2012).

A concentration of 0.5 mg/l of BPA increased the number of flowering plants (Fig. 4H) and a BPA concentration higher than 0.5 mg/l might have a less stimulatory effect as the mean number of flowering plants decreased in a dose-dependent manner in comparison to those plants treated with 0.5 mg/l. However, these results were not statistically significant. Still, the mean values of the flowering rate were higher in BPA-treated plants. Therefore, we cautiously assume that BPA may have a positive effect on flowering, but further investigation is essential to gather more evidence for that thesis. According to our knowledge such effect was not previously observed. On the contrary, some evidence suggests the opposite effect as a very low BPA concentration (as low as 10 nM – less than 0.01 mg/l) inhibits flowering in *A. thaliana* (Tian *et al.*, 2014). Stress conditions often accelerate flowering in plants. However, in the case of our study no obvious stress symptoms occurred. Hence, it seems that BPA might indeed induce generative phase in *A. thaliana*. Importantly, exogenous estrogens were shown to induce flowering in particular plant species (Kopcewicz, 1970b). It is an open question, whether BPA, an estrogene-mimicing substance, could possibly act in a similar way. Some evidence suggests that BPA may increase the endogenous production of steroid hormones (17 β-estradiol and testosterone, in particular) in kiwifruit (*Actinidia deliciosa*) pollen and thus inhibit its germination (Speranza *et al.*, 2011). Estrogens also have a negatively affect on auxin content (Kopcewicz, 1970a). Notably, auxins and ethylene are hormones involved in the control of flowering and it was suggested, that BPA may interfere negatively with auxin regulation, as well as decrease the expression of particular ethylene- and auxin-dependent genes (Tian *et al.*, 2014; Frejd *et al.*, 2016).

BPA is and probably still will be one of the most ubiquitous environmental pollutants. Even with limitations, there is a risk of the influential effect of BPA on the environment. Our findings indicate that BPA induces unexpected changes in plant physiology and biochemistry. Thus, further research on the role of a low BPA concentration on plant physiology is essential.

CONCLUSIONS

A low concentration of BPA (up to 5 mg/l) does not induce oxidative stress symptoms and photoinhibition in *A. thaliana*.

BPA at a concentration of 5 mg/l has a stimulatory effect on chlorophyll and carotenoid accumulation in one-week-old seedlings of *A. thaliana*.

BPA does not affect PSII activity and overall photosynthesis efficiency in 4-week-old plants.

A low concentration of BPA (up to 5 mg/l) decrease protein content in 4-week-old plants.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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